# Herpesviral Interactomics

*Intraviral and virus-host protein-protein interaction network from different species of herpesviruses*

**Even Fossum**


Dissertation for the degree philosophiae doctor (PhD)

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*Til mamma og pappa*

*"Nothing shocks me. I'm a scientist."*
*Indiana Jones*

# Scientific environment

This thesis was initiated in 2004 and conducted at the Max von Pettenkofer Institute, Ludwig Maximilians Universtät, Munich, Germany. Financial support was provided by the Bayerisches Genomforschungsnetzwerk.



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Even Fossum

Edinburgh, 28.09.2007

# Contents

# Abbreviations

| | |
|---|---|
| **3-AT** | **3-amino-1, 2, 4-triazole** |
| **AD** | **Activator domain** |
| **AIDS** | **Acquired Immune Deficiency Syndrome** |
| **BD** | **Binding domain** |
| **BMT** | **Bone Marrow Transplant** |
| **Bp** | **basepair** |
| **CHO** | **Chinese Hamster Ovary** |
| **CNS** | **Central Nervous System** |
| **CR2** | **Complement receptor 2** |
| **CTL** | **Cytotoxic T lymphocytes** |
| **DC-SIGN** | **dendritic cell-specific ICAM3-grabbing nonintegrin** |
| **DCTN1** | **Dynactin 1** |
| **DNA** | **Deoxyribonucleic acid** |
| **EBNA1** | **EBV Nuclear Antigen 1** |
| **EBV** | **Epstein Barr virus** |
| **EGFP** | **Enhanced green fluorecent protein** |
| **EHV** | **Equine herpesvirus** |
| **FRET** | **Förster Resonance Energy Transfer** |
| **GAL4** | **DNA-binding transcription factor required for the activation of the GAL genes in response to galactose; repressed by Gal80p and activated by Gal3p** |
| **Gar** | **Glycine-Aanine repeats** |
| **gB** | **Glycoproteins B** |
| **H2A** | **Histone 2B** |
| **H3K9me** | **Methylation of histone 3 lysine 9** |
| **HCMV** | **Human cytomegalovirus** |
| **HHV** | **Human herpesvirus** |
| **HIV** | **Human immunodfency virus** |
| **HP1** | **Heterochromatin binding protein 1** |
| **HSV** | **Herpes simplex virus** |
| **HTATIP** | **HIV-1 Tat interacting protein, 60kDa** |
| **HVEM** | **Herpesvirus Entry Mediator** |
| **IDE** | **Insulin Degrading Enzyme** |
| **IFN-γ** | **Interferone gamma** |
| **IKBKAP** | **Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase complex-associated protein** |
| **IKK** | **IκB kinases** |
| **ING5** | **Inhibitor of growth family, member 5** |
| **Kbp** | **kilobasepair** |
| **KS** | **Kaposi's sarcoma** |
| **KSHV** | **Kaposi's sarcoma associated herpesvirus** |
| **LANA1** | **Latency Associated Nuclear Antigen 1** |

| | |
|---|---|
| **LAT** | **Latency Associated Transcript** |
| **LMP2A** | **Latent Membrane Protein 2A** |
| **LUMIER** | **Luminescence-based mammalian interactome mapping** |
| **mCMV** | **Murine cytomegalovirus** |
| **miRNA** | **micro RNA** |
| **MS** | **Mass spectrometry** |
| **MYST2** | **MYST histone acetyltransferase 2** |
| **NEC** | **Nuclear Egress Complex** |
| **NF-κB** | **Nuclear factor of kappa light chain gene enhancer in B-cells** |
| **NIK** | **NF-κB-inducing kinase** |
| **ORF** | **Open Reading Frame** |
| **PEL** | **Perfusion Effusion Lymphoma** |
| **PEL** | **Primary effusion lymphoma** |
| **pp150** | **Phosphoprotein/UL32** |
| **pp65** | **Phosphoprotein/UL83** |
| **PPI** | **Protein-protein interaction** |
| **PrV** | **Pseudorabies virus** |
| **RNA** | **Ribonucleic acid** |
| **RPA 1** | **replication protein A1** |
| **siRNA** | **Silencing RNA** |
| **SMAD3** | **mothers against decapentaplegic homolog 3** |
| **SOT** | **Solid Organ Transplant** |
| **TAP** | **Tandem Affinity Purification** |
| **TGF-β1** | **Transforming growth factor, 1 beta** |
| **THBS1** | **Thromospondin 1** |
| **UL** | **Unique long** |
| **US** | **Unique short** |
| **VZV** | **Varicella Zoster virus** |
| **xCT** | **solute carrier family 7, (cationic amino acid transporter, y+ system) member 11** |
| **Y2H** | **Yeast-Two-Hybrid** |

# 1 Introduction

## *1.1 Herpesviridae*

The *Herpesviridae* comprises a large family of enveloped double stranded DNA viruses, with a broad host spectrum ranging from mammals to birds and reptiles. More than 100 different species of herpesviuses have been identified, including 9 human pathogenic viruses. Common for all herpesviruses are the ability to persist within a host in a latent state after primary infection. During latency only a few viral genes are expressed, thus limiting the host's opportunity to establish an immune response directed against specific viral antigens. The latent virus can reactivate at later time points and lead to secondary infections which sometimes are of a different nature than the primary infection.

### 1.1.1 Subfamilies and phylogeny

*Herpesviridae* is divided into three different subfamilies, the alfa-, beta- and gamma-herpesvirinae. The three subfamilies were initially separated based on biological differences such as cell tropism and growth properties in cell-culture. *Alfaherpesvirinae* are neurotrophic and replicate efficiently in cell culture, whereas *Betaherpesvirinae* have a narrower cell tropism in culture and infection in-vivo may results in enlargement of the infected cells (cytomegalia). *Gammaherpesvirinae* replicate poorly in culture and are oncogenic lymphotropic viruses. With the advances in genetics, herpesviruses are now assigned to subfamilies based on genomic differences. While most of the biological differences accurately predict the subfamily association, some viruses have been moved into a different subfamily after their genome was fully sequenced. This was the case for Marek's disease virus (MDV), which was initially thought to be closely related to Epstein Barr virus (EBV) (a gammaherpesvirus) due to its ability to infect lymphocytes in addition to its oncogenicity [1]. Genetic analysis however revealed that the virus had more in common with alfaherpesviruses [2-4]. The evolutionary divergence of the three subfamilies has been predicted to have taken place around 400 million years ago (Figure 1.1), which is about the same time as prehistoric animals first started venturing onto land [5, 6].

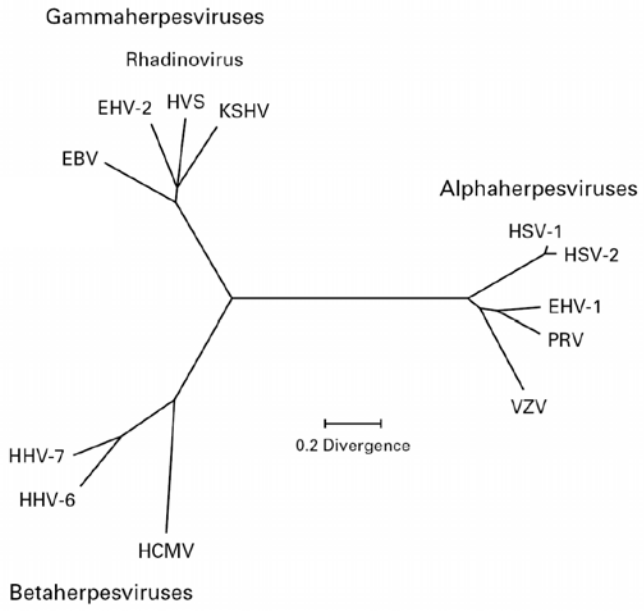


*Figure 1.1: Phylogeny of the Herpesviridae. The tree represents the relationship between all known human pathogenic herpesviruses (for further description see 1.1.2), in addition to Herpesvirus Saimiri (HVS), Equine herpesvirus type 1 and 2 (EHV) and Pseudorabies virus (PrV). Major branches on the tree represent the three subfamilies alfa-, beta- and gamma-herpesvirinae. Illustration modified from Antman et al. 2000 [7].*

With the recent identification of two evolutionary distinct classes of herpesviruses, one infecting the Pacific oyster and other bivalved hosts [8], and one infecting bonefish and amphibians [9-11], a new herpesvirus taxonomi has been suggested. The order now known as *Herpesviridae* would be renamed *Herpesvirales*, with *Herpesviridae* as one of three families in this order. *Alloherpesviridae* (fish and amphibians) and *Malaoherpesviridae* (oysters) would make up the other two families in the new order. While the evolutionary relationships of single species within the different families can be defined, the time point from which the three families have diverged is not yet known [12].

### 1.1.2 Human herpesviruses and their clinical implications

There are currently identified 9 different human herpesviruses, distributed over all three subfamilies of the *Herpesviridae*. Herpes Simplex virus-1, -2 (HSV-1 and -2) and Varicella zoster virus (VZV) belong to alfaherpesvirinae, while human cytomegalovirus (HCMV), in addition to Human herpesvirus-6A, -6B and -7 (HHV-6 and -7) are classified as betaherpesvirinae. Epstein Barr virus (EBV) and Kaposi's sarcoma associated herpesvirus (KSHV) belong to the gammaherpesvirinae. Most of the viruses have a relatively high

prevalence in humans, with HSV-1, VZV and EBV among the most ubiquitously distributed. HSV-1 is reported to have a sero-prevalence of 40 – 90 % [13-15], while VZV and EBV generally have ~ 90 % prevalence [16, 17]. The numbers vary depending on geographical location and which age groups are studied.

Herpesviruses are involved in a number of different diseases in humans, with alfaherpesvirus infections being the most extensively studied. Herpes Simplex-1 and -2 can cause recurrent infections of mucous membranes, with HSV-1 generally giving oro-facial lesions and HSV-2 giving genital lesions [18]. While such infections interfere with an individual's quality of life, they are seldom life-threatening. In some cases however, Herpes Simplex virus infection can result in more serious illnesses, like ocular infections [19, 20] or meningitis and encephalitis [21]. VZV is the causative agent of the childhood disease varicella (popularly called chickenpox). During primary infection the patient experiences fever and vesicular rash. Later reactivation of the virus can lead to herpes zoster and in some cases chronic pain in the form of post herpetic neuralgia [22].

Human cytomegalovirus (HCMV) primary infections are in most cases asymptomatic, both in children and adults. It may however occasionally result in symptoms resembling a mild infectious mononucleosis with fever, myalgia and lymphadenopathy. In contrast, congenital HCMV infections of neonates represent a serious threat, and are connected to severe disease manifestations like microcephaly, chorioretinitis, nerve deafness and hepatitis [23]. Congenital HCMV infections occur in 0.5 – 1 % of all live births, with the majority of these infections being asymptomatic at birth. Although there can be a certain degree of morbidity connected to asymptomatic infections, the chances of severe illnesses are higher when the babies are symptomatic at birth. In addition to HCMV, congenital infections from HSV and VZV are also relatively frequent (ranging from 1 in 3200 up to 1 in 60.000 for HSV and 5 in 10.000 for VZV [24, 25]), with local infections of the central nervous system (CNS) and disseminated infections involving CSN and other organs having the highest morbidity and mortality [26].

Most EBV infections are though to occur during childhood, resulting in sub-clinical infections. However, if primary infection is delayed until adolescence there is a substantial risk of developing mononucleosis with the possibility prolonged malaise including pahryngitis and lymphodenopathy [27]. As previously indicated, EBV is an oncogenic virus,

and although the number of people affected by EBV associated tumors in western countries are quite low, the situation is very different in parts of the developing world. The virus is thought to be associated with Burkitt's lymphoma, which is one of the most common cancers affecting young children in equatorial Africa. In addition, EBV has also been linked to Hodkin's disease and nasopharyngeal carcinoma [27]. KSHV generally has a low sero-prevalence in North America and Europe (~ 5 %) [28, 29], with southern Italy and other Mediterranean countries having a somewhat higher prevalence [30, 31]. In sub-Saharan Africa, however, KSHV can be considered common with sero-prevalence ranging from 37,5 in Zambia [32], up to 90% in Botswana [33]. Primary infection with KSHV has so far only been connected to mild and unspecific symptoms of diarrhea, fatigue, rash and lymphadenopathy [34]. Similar to EBV, KSHV has been connected to several forms of tumors in man. Kaposi's Sarcoma (KS) was first described by Moritz Kaposi in the 1870s, but was only connected to KSHV as late as in 1994 by Chang and colleagues [35]. In addition to KS, which probably has a lymphatic endothelial or blood vascular endothelial origin [36], KSHV is also associated with the B-cell lymphomas Primary effusion lymphoma (PEL) and Multicentric Castleman's disease (MCD) [37, 38].

Although Kaposi's Sarcoma can be found in healthy individuals, then referred to as classic KS, both the occurrence of KS and the sero-prevalence of KSHV is substantially higher in people infected with human immunodeficiency virus (HIV) [39] [40]. Acquired Immune Deficiency Syndrome (AIDS) related KS is one of the most common malignancies found in AIDS patients, and is connected to increased mortality [41]. Other herpesviruses have also been connected to disease manifestations in AIDS patients, with increased risk of VZV herpes zoster and HCMV retinitis being two such examples [42, 43]. The drastic increase of KS in HIV positive individuals is believed to be a result of immune suppression associated with the HIV infection. However, immune suppression can also be a result of other circumstances. With the increase in organ transplant patients, and the subsequent treatment with immunosuppressive drugs, diseases related to herpesvirus infections are becoming more frequent. HCMV has been extensively reported to cause complications in both solid organ transplant (SOT) and bone marrow transplant (BMT) recipients. For renal transplant recipients, HCMV infection has been associated with increased graft rejection and renal artery stenosis [44, 45]. HCMV infections as a result of BMT may lead to pneumonitis, which can have a mortality rate as high as 80 % if left untreated [46]. Also HSV and VZV are associated with common complications of organ transplantations, mainly as a result of reactivation of

latent virus. HSV hepatitis has been connected with both SOT and BMT, where transplant recipients have a poor clinical prognosis if not treated early [47, 48] The incidences of herpes zoster as a result of VZV reactivation in individuals receiving SOT is significantly higher than in the general population. However, with early onset of antiviral therapy there is a low likelihood of visceral dissemination [49].

### 1.1.3 Structure

Herpesvirus infectious particles have a diameter of approximately 150 - 200 nm, and can be divided into four separate structural elements: core, capsid, tegument and envelop (Figure 1.2a and 1.2b). The core consists of the viral genome, which is believed to be packaged in a toroid shape [18, 50]. While all herpesviral genomes are made up of double stranded DNA, the genome size of different species can vary from 125 kbp (VZV) up to 230 kbp (HCMV) [51, 52]. The genome size also reflects the protein coding potential of different species with VZV encoding 72 open reading frames (ORFs), while HCMV encodes approximately 200 ORFs. In addition, the genetic organization of the genomes differs between species. While the HSV-1 genome consists of two covalently linked segments called Long (L) and Short (S), each flanked by sequences of inverted repeats, the KSHV genome is made up of only one segment flanked by repeats [53].

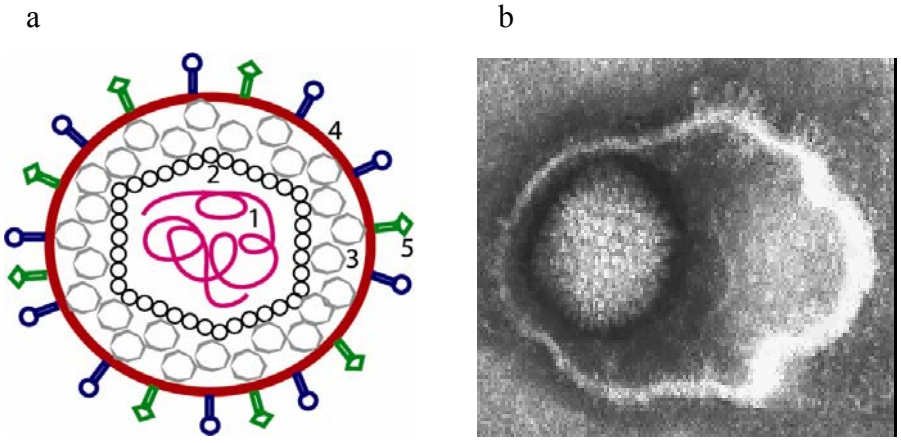


*Figure 1.2: Schematic overview of the herpesvirus infectious particle. a) The virus core consists of the vial genome (1) packaged within the capsid (2). The tegument (3) surrounds the capsid, and separates it from the outer membrane (4). Imbedded in the membrane are glycoproteins that protrude out from the virus particle (5). b) Electron micrograph of a Herpes Simplex 1 particle (Electron micrograph obtained from http://www.ncbi.nlm.nih.gov/ICTVdb)*

The viral capsid structure is an icosahedric protein shell made up of 162 capsomers [18]. Of these, 12 are pentavalent capsomers located at the vertices of the capsid, while the remaining 150 are hexavalent capsomers [54-56]. Surrounding the capsid is a more loosely structured tegument layer which contains more than 15 proteins in the case of HSV-1 [57]. While the composition and structure of the capsid is quite conserved throughout the herpesviridae, the composition of the tegument has a higher degree of variation between different species. There are however a set of at least 7 tegument proteins which are believed to be conserved between the three subfamilies [12]. This claim is backed up by mass spectrometry analysis of purified virion particles, which has confirmed the presence of many of these proteins in several species of herpesvirus [58-62]. The viral envelop is a double lipid layer which surrounds and contains the tegument proteins. Embedded in the viral envelop are a variety of transmembrane glycoproteins. HSV-1 reportedly contain at least 12 different glycoproteins designated gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, gM and gN. Of these at least 5 are conserved between the three subfamilies [12].

### 1.1.4 Infection, replication and egress

Herpesvirus infection initiates when one or more of the glycoproteins protruding from the viral envelop attach to specific surface receptors on the host cell. Different herpesvirus species will attach to different surface receptors, which is one important factor in determining the tropism of a specific virus. Cell-lines that originally are non-permissive for a specific herpesvirus, can be rendered permissive by expressing the right surface receptor in trans. This has been observed for HSV-1 where the cell-line CHO-K1 (Chisese Hamster Ovary), which normally does not support replication of the virus, becomes permissive when transfected with the cellular receptor HVEM (Herpesvirus Entry Mediator) [63]. In addition to HVEM, HSV-1 can also attach to the cellular surface proteins nectin-1 and nectin-2 [64, 65], although nectin-2 is probably more active for HSV-2 [66]. There is also evidence that the virus utilizes different receptors depending on the cell being infected [67, 68]. Other cellular receptors for herpesviruses include Insulin degrading receptor (IDE) for VZV [69], intergrins for HCMV [70, 71], complement receptor 2 (CR2) for EBV [27], and DC-SIGN, xCT and Intergrin α3β1 for KSHV [72-74].

Several theories describing possible mechanisms of how the viral capsid enters the host cell have been proposed. The most accepted theory suggest that the interaction between the viral

glycoprotein and cellular receptor brings the viral envelop into close proximity of the cellmembrane, resulting in a fusion between the two membranes [18]. Viral tegument proteins and capsid are then released into the host-cell, thus initiating the infectious cycle. However, there are also indications that other routes of viral entry are taking place *in vivo*. For both HSV and HCMV there are reports indicating that the viruses can be endocytosed by the host cell, and that this also results in a productive infection [75-77]. This proposed mechanism of herpesviral entry is however quite recent, so many of the detail are not yet thoroughly studied, including how the virus is able to exit the endocytic vesicles once inside the cell.

Once released into the cytoplasm, the capsid is actively transported to the nucleus along microtubule, and this transport is believed to be dependent on the motorproteins dynein and dynactin [78, 79]. Once there, the viral genome is transported through the nuclear pore leaving behind the empty capsid. Studies using temperature sensitive mutants of HSV-1 have indicated that the large tegument protein (UL36 for HSV-1) is involved in this process [80]. After the viral genome enters the nucleus, transcription of the viral genes occurs in a cascade-like manner where the immediate early (IE) genes are expressed first, followed by the early (E) genes and finally the late (L) genes. While the IE genes mostly encode transcriptional activators necessary for proper expression of E and L genes, E genes encode genes involved in the replication of the viral genome. Late genes are first expressed after replication of the viral genome is initiated, and encodes to a large degree structural proteins required for making the viral particles [18].

Production of new virus particles is initiated within the nucleus of the infected cell, in specific replication compartments [81, 82]. These compartments are though to contain the structural proteins which make up the capsid, the proteins necessary for replicating the viral DNA, in addition to other proteins necessary for proper production of new viral particles. One of these proteins is the viral scaffolding proteins (UL26 for HSV-1), which forms a scaffold for the capsid proteins to assemble around [26]. The capsid structure is made up of four separate proteins thought to be conserved throughout the herpesviridae. Herpesviral DNA is replicated in a rolling circle mechanism, resulting in a long concatomeric DNA molecule where several viral genomes are organized in a head-to-tail fashion. The concatomoeric DNA molecule is subsequently cleaved into single genome fragments during the packaging process [26]. Packaging of newly replicated viral DNA is first initiated after the capsid-scaffold structure is constructed. It is believed that once DNA start entering the scaffold, the pH within the pre-

capsid is lowered resulting in a self-cleaving of UL26 which then diffuses out through pores in the capsid structure [26]. For HSV, six viral proteins has been reported to be essential for proper packaging of the viral genome, including UL6, UL15, UL17, UL28, UL32, UL33 [83-88]. These proteins have been designated as packaging proteins since deletions or mutation of any of these genes may result in partial or no packaging of the viral DNA. The two proteins UL15 and UL28 make up the terminase complex, and have been reported to be involved in cleavage of the concatomeric DNA as it is package into the capsid [89]. It is also been suggested that UL33 is a part of the terminase complex, and that it interacts with UL28 and stabilizes the UL15/UL28 complex [90, 91].

After packaging of the viral DNA, the finished capsid is believed to acquire an initial tegument layer within the nucleus. The composition of this initial tegument is however not known. The capsid is subsequently transported through the inner nuclear membrane and into the perinuclear space, obtaining an initial viral envelop in the process. Two viral proteins, UL31 and UL34 in HSV-1 (labeled the nuclear egress complex (NEC)), has been reported to play an important role in the nuclear egress of herpesviruses [92]. Orthologs of these proteins has also been reported to share similar function in Pseudorabies virus (PrV) [93], mCMV [94] and EBV [95]. It is believed that the initial viral envelop is lost when the viral particle fuses with the outer nuclear envelop, releasing the uncoated virus into the cytoplasm. For HSV, the viral kinase US3 is reported to play an important role in this process, due to the observation that US3 deletion mutants accumulate in the perinuclear space [96]. Once released into the cytosol, the viral particle acquire its final tegument, and goes through a second envelopment when it is transported into the trans-golgi [97, 98]. The details of how the viral particle is transported into the golgi is not fully known, but for PrV and EHV-1 (Equine herpesvirus 1) the conserved proteins gM and UL11 has been reported to play an important role [99-101]. Final egress out of the infected cell goes through sectretorial vesicles budding off from the golgi.

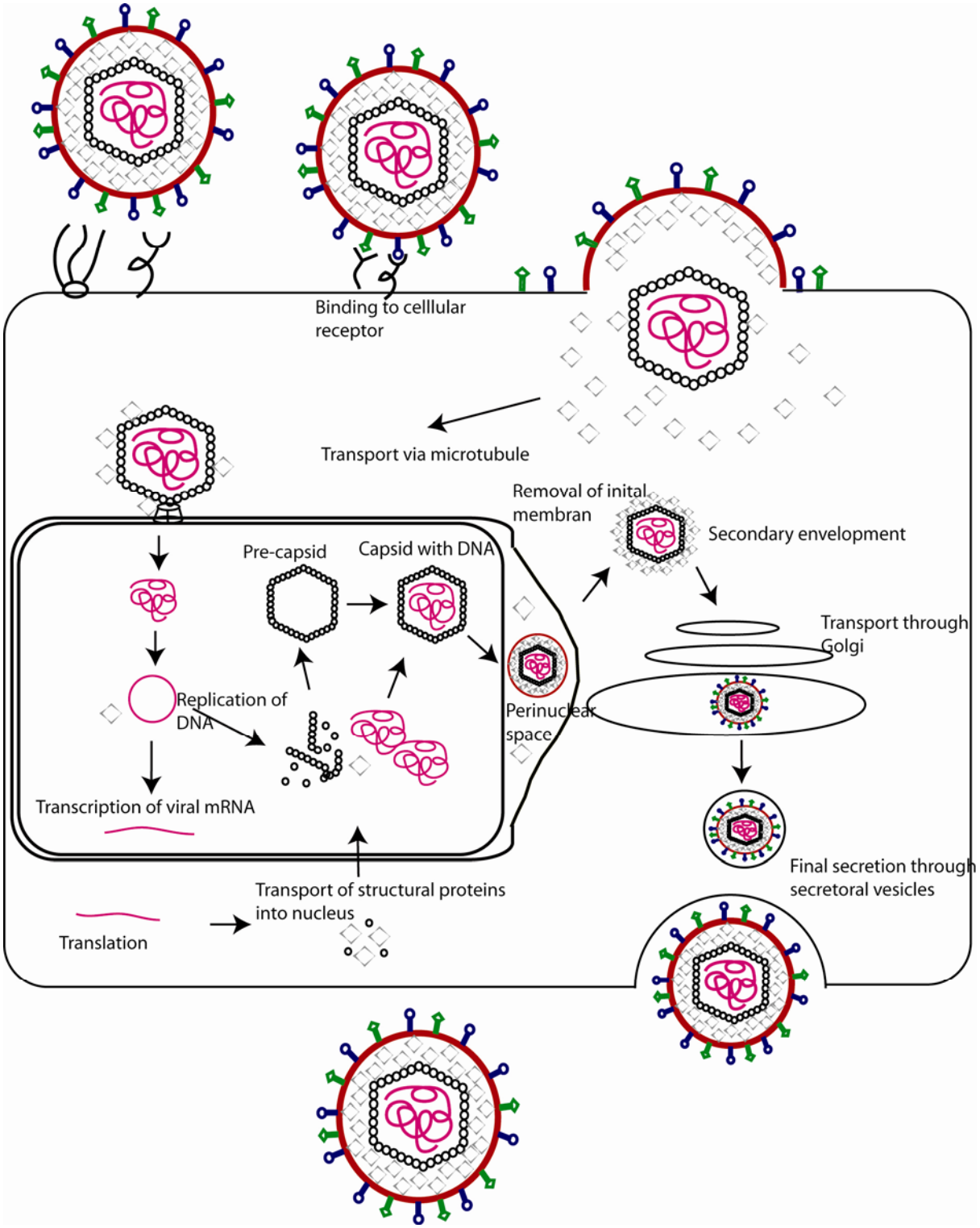


*Figure 1.3: Overview of the infectious cycle of herpesviruses. The viral particle attaches to receptors on the cell surface which leads to a fusion between the viral envelop and the cellular membrane. Once released inside the cell the capsid is transported along the*

*microtubule to the nuclear membrane, where the viral genome is transported into the nucleus through the nuclear pore. The viral genes are expressed in a cascade-like fashion with IE genes generally being transcriptional regulators controlling the expression of the E and L gene classes. After replication of the viral genome is initiated, the structural proteins necessary for generating new infection particles is expressed. Newly replicated DNA is packaged into preformed capsids which are subsequently transported from the nucleus to the perinuclear space through the inner nuclear membrane. During this process the virus gains an initial viral envelope which is subsequently lost when the virus particle buds through the outer nuclear membrane. In the cytoplasm the virus particle recruits its final tegument, and receives is final envelop when transported into the trans-golgi network. The viral particle is transported out of the cell through secretarial vesicles.*

### 1.1.5 Latency

All herpesviruses are able to enter a latent stage of infection where only a few genes are expressed. By limiting the number of proteins expressed the virus can minimize the viral epitopes presented by class I MHC, and thus prevent detection by cytotoxic T-lymphocytes (CTL). Latently EBV infected B-cells from healthy individuals generally express only two viral proteins, LMP2A (Latent Membrane Protein) and EBNA1 (EBV Nuclear Antigen) [102, 103]. Even though the proteins are expressed, they do not simulate any significant CTLs response. EBNA1 manages this through a domain of glycine-alanine rich repeats (GAr) which inhibits its proteasomal degradation, and subsequent peptide presentation. The exact mechanism of how EBNA1 escapes the proteasome is not fully elucidated, but it has been suggested that the GAr binds to the 19S cap of the 26S proteasome and prevents further processing of ubiquiniated substrates [104]. LMP2A also shows resistance to epitope presentation through the proteasome pathway which can be related to specific amino acids in its C-terminal domain [105]. This resistance can however be abrogated *in vitro* if cells are exposed to INF-γ [106]. A similar situation can be seen for HCMV, where the most immunodominant CTL epitopes are directed against a few proteins expressed during the lytic cycle [107, 108].

During latency the viral genome is attached to the host chromosomes, and is replicated with the host chromosomes during mitotic cell-division [26, 53]. For KSHV it has been reported that LANA1 (Latency Associated Nuclear Antigen 1) is responsible for tethering the viral

genome to the cellular chromosomes through binding to histones H2A-H2B [109]. EBNA1 has a similar function to LANA1 in EBV, and is reported to attach to host chromosomes through its N-terminal domain [110]. While there are reports indicating that EBNA1-mediated persistence of the viral genome is dependent on interactions with EBP2 (the chromosome associated protein EBNA Binding Protein 2) [111, 112], this has been disputed by others [110].

As previously mentioned, there are only a few viral genes expressed during latency, with the majority of genes only being expressed when the virus enters the lytic replication cycle. This is reflected in the epigenetic regulation of lytic genes which, during latency, have a condensed chromatin structure (heterochromatin), in accordance with transcriptional silencing [113, 114]. One of the hallmarks of heterochromatin formation is methylation of lysine 9 on histone 3 (H3K9me), and recruitment of heterochromatin binding proteins 1 (HP1) [115, 116]. Acetylation of H3, on the other hand, is located at sites of transcriptional activation and a more open chromatin structure [117]. Ioudinkova and colleagues have shown that HCMV lytic genes, like the viral polymerase (UL54), pp65 (phosphoprotein/UL83) and pp150 (phosphoprotein/UL32), are indeed methylated on H3 during latency, and acetylated on H3 when expressed in the lytic cycle [118]. Similar results have been shown for HSV, where the LAT (Latency associated transcript) has been reported to be important for heterochromatin formation on lytic genes during latency [119]. For KSHV, it has been shown that demethylation of the ORF 50 promotor, ORF 50 being the lytic transactivator RTA, is enough to change the infection from a latent to lytic state [120].

More recently it has been reported that some herpesvirus species also express micro-RNAs (miRNAs) during latency [121, 122]. miRNA are short (~22 nucleotide) RNA transcripts involved in gene regulation, that can bind to specific mRNAs and inhibits its further translation. HSV-1 encodes a miRNA within its LAT-transcript, and KSHV encode at least 12 miRNA within its K12 region [121-124]. Although the target mRNAs for most of these miRNA are still missing, HSV-1 LAT miRNA has been reported to inhibit apoptosis of infected cells through down-regulation of TGF-β1 (transforming growth factor, 1 beta) and SMAD3 [122]. A recent study from KSHV has also suggested several targets for the 12 miRNAs, including the tumor suppressor gene thromospondin 1 (THBS1) [125].

### 1.1.6 Conserved and non-conserved genes

Herpesviruses encode a set of orthologous genes, or core genes, which can be found in members of all three subfamilies. The published number of conserved genes varies from ~ 26 to ~ 40 depending on the stringency of the methods used by the authors [12, 126-128]. When only taking sequence similarity into consideration the number of conserved genes is limited to around 30. However, several proteins are believed to share similar function although they show a very low sequence similarity. This is especially true for some of the structural proteins that make up the capsid. When these proteins are included the number of core proteins rises to approximately 40. The herpesviral core protein are generally cluster in the center of the viral genome (Figure 1.4), and are involved in the fundamental aspects of viral replication (e.g. DNA replication, DNA packaging, structure and egress). This is reflected in that a large portion of the core genes are essential for the virus to replicate in cell culture [129-131]. Genes involved in immune escape generally encode non-core proteins, which often are non-essential for growth in cell culture. They can however be required for the virus to properly infect and replicate in a host organism.

While core proteins are derived from an ancestral herpesvirus, before the separation into three distinct subfamilies, the remaining proteins encoded in the viral genome are divided into subfamily and species-specific genes most likely acquired more recently. About 13 % of the herpesviral genes have been found to have sequence similarity to human genes [132]. The majority of these proteins are species or sub-family specific, with the exception of four core poteins (including ribonucleotide reductase large subunit (HSV-1 UL39), uracil-DNA glycosylase (HSV-1 UL2), helicase/primase (HSV-1 UL5) and DNA polymerase (HSV-1 UL30)).

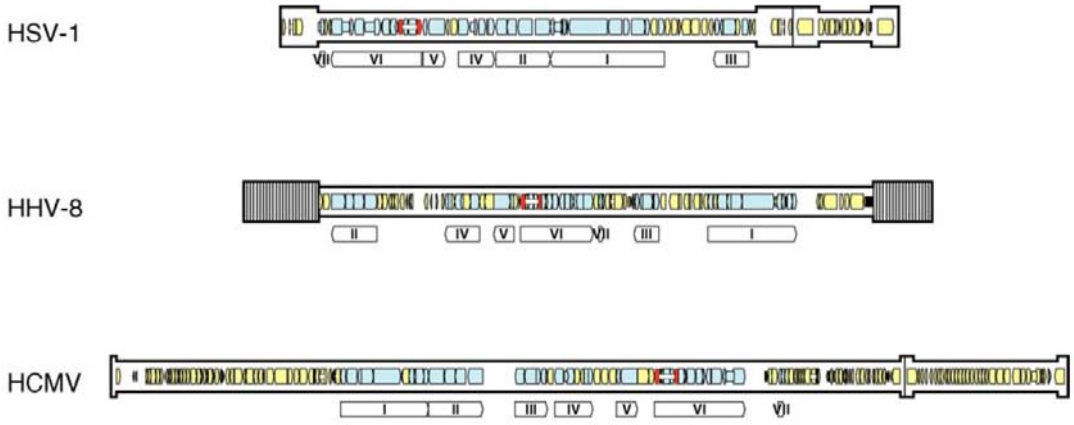


*Figure. 1.4 Genomic organization of conserved genes in herpesviruses, represented by HSV-1, KSHV and HCMV. Protein coding regions corresponding to core genes are shown in blue color, while non-conserved genes are colored yellow. The core genes are divided into seven conserved geneblocks labeled I-VII, which are differentially organized in the different subfamilies. Introns are in white color, and the ATPase subunit of the terminase complex, the only gene also conserved between Herpesviridae, Alloherpesviridae and Malaoherpesviridae, is indicated in red. Modified from* [133]

## *1.2 System biology*

### 1.2.1 Definition of system biology

Systems biology is the study of the interactions between the components of a biological system, and how these interactions give rise to the function and behavior of that system (definition taken from Wikipedia (http://www.wikipedia.org)). While the ultimate goal of systems biology is to give a complete and accurate model of how all the different components of a cell behave and interact with each other, current studies are generally limited to examining one type of component (e.g. protein or RNA) or one defined type of interaction (e.g. protein-protein or enzyme-substrate) at a time. This has spawned several sub-disciplines that all fall within the category of systems biology: interactomics which focuses on protein-protein or protein-DNA interactions [134], proteomics which focuses on identifying and quantifying proteins (mainly through mass spectrometry) [135], transcriptomics which look at expression of RNA and how this is influenced by internal and external factors [136], and metabolomics which detects and measures levels of different metabolites [137]. Common for all these fields is that studies often produce large quantities of data which require further processing using different bioinformatics tools. Advances in bioinformatics has also resulted in the possibility of combining data obtained from different sub-disciplines, resulting in a more complete picture of what goes on in a cell or an organism [138].

### 1.2.2 Yeast-two-hybrid

Yeast-two-hybrid (Y2H) as a method for looking at protein-protein interactions was first described in 1989 by Fields and Song [139]. They divided the transcription factor GAL4 (DNA-binding transcription factor required for the activation of the GAL genes in response to galactose; repressed by Gal80p and activated by Gal3p**)** into two separate domains, its DNA binding domain (DB) and transcription activating domain (AD), and then fused different proteins to these domains. If protein A fused to DB interacts with protein B fused with AD the complete transcription factor is reconstituted allowing the transcription of a reporter gene. Theoretically any reporter gene can be used to visualize an interaction between proteins A and B, but the HIS3-gene reporter system is one of the most widely used. By placing the HIS3 gene, encoding imidazoleglycerol-phosphate dehydratase which is essential for yeast to

synthesize histidine, it is possible to identify interacting proteins based on the yeast clones ability to grow in media lacking histidine [140].

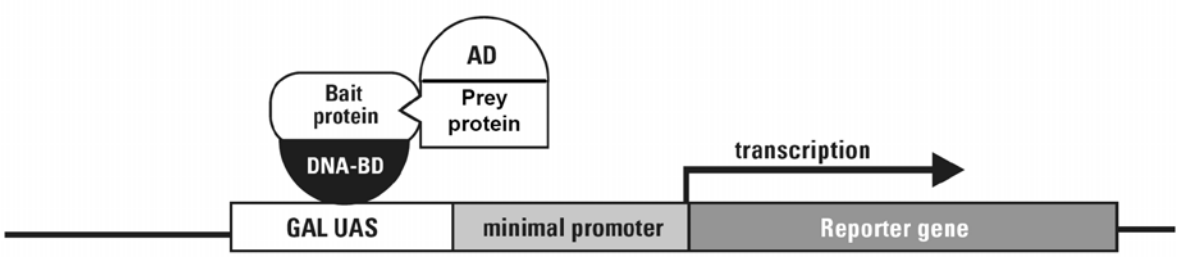


*Figure 1.5 Principle of Yeast-two-hybrid. A protein of interest is fused to the DNA binding domain of the GAL4 transcription factor (referred to as the bait protein), while a second protein is fused to the activator domain of GAL4 (referred to as prey protein). If the prey and bait protein interact they reconstitute the GAL4 transcription factor which induces the transcription of a reported gene. (Modified from the Matchmaker GAL4 Two-Hybrid System 3 & Libraries User Manual (Clontech))*

Two different approaches can be used when identifying protein-protein interactions by yeast-two-hybrid, the matrix screen and the library screen. The matrix screen approach requires that you have a fixed set of known proteins to be tested against each other for the ability to interact, and that you have all the proteins as fusion constructs with either the AD (prey) or the BD (bait) domain (or preferentially both). For a set of 10 proteins, protein 1 as prey can then be tested individually against proteins 2-10 as baits. Since the identity of all the proteins tested are known, it is possible to immediately identify the interacting proteins. In a library screen approach a single protein as bait is analyzed against a library of prey proteins. Since the identity of the interacting prey is not known in a library screen, all positive interactions have to be sequenced to identify the prey proteins. Generally, a matrix screen is faster and cheaper since positive samples do not have to be sequenced. However, the need to clone each protein to be tested in a matrix screen separately makes it less convenient to use in genome-wide studies. While a library screen allows you to screen for more possible interactions, the quality of the analysis is heavily dependent on the quality of the prey libraries.

While currently the most commonly used method for large-scale analysis of PPIs, Y2H screens are troubled by the presence of false negative and false positive interaction. False negative interactions are PPI that occur in the cell, but fail to be detected by the Y2H analysis,

and affect the coverage of the analysis. In contrast, false positive interactions are not observed in the cell, but are still found to be positive in the Y2H analysis, resulting in lower specificity. The false positive rate in Y2H have been reported to be around 50 %, and remains a serious problem in generating reliable data using this technique [141, 142]. This problem can however be addressed by retesting interactions using a second independent analysis, with variants of co-immunoprecipitation being among the most commonly used techniques. False negative interactions are however more difficult to assess, since one would need a complete set of verified interactions to know how many are being missed. It is however apparent from comparative studies that there is generally little overlap between large-scale Y2H studies, indicating a high degree of false negative interactions [143-145].

### 1.2.3 Large-scale interactome studies

The first two organisms analyzed, on a genome-wide level, for protein-protein interactions were the bacteriophage T7 virus and Vaccinia virus [146, 147]. Both of which have relatively small genomes encoding few proteins. However, recent advances made to the Y2H technique, as well as the introduction of robotics, have made it possible to screen large number of proteins against each other. This resulted in the first large-scale interactions network (also referred to as an interactome) for *Saccharomyces cerevisiae* being published by Uetz et al in 2000 [148]. In addition to the publication of a more comprehensive study of interactions in yeast [149], large scale interaction maps have also been generated for *Helicobacter pylori* [150], *Caenohabditis elegans* [151], *Drosophila melanogaster* [152], *Plasmodium falciparum* [153] and *Homo sapiens* [154, 155].

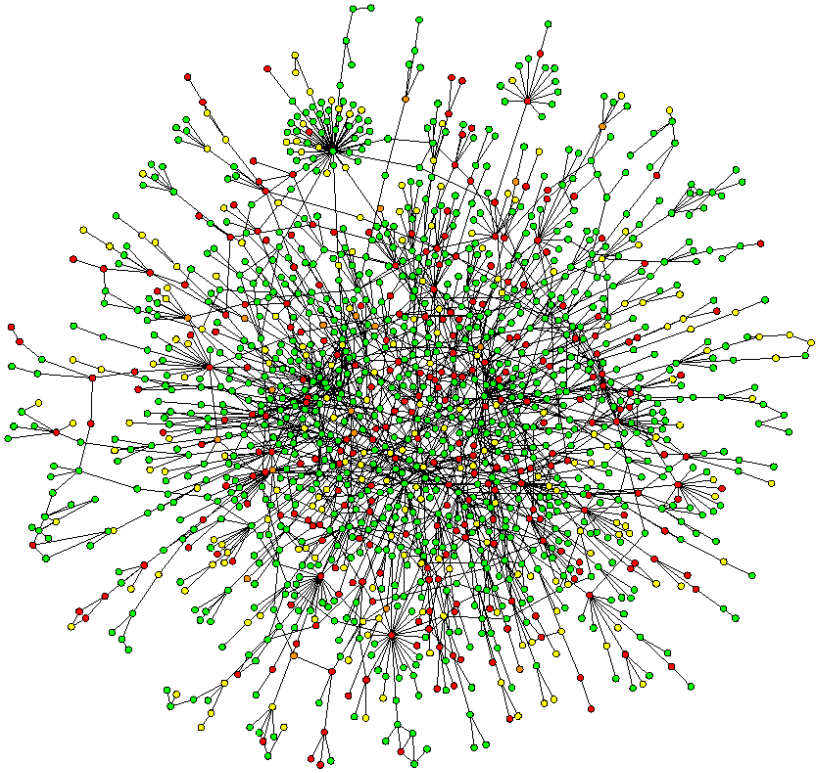

*Figure 1.6: Map of protein-protein interactions in Saccharomyces cerevisiae. Circles represent proteins (nodes), and the lines between them (edges) indicate an observed interaction. The colors refer to the phenotypic effect observed when deleting a specific protein. Removal of a red node is lethal for the yeast, green are non-lethal, orange result in slower growth and yellow are unknown. Modified from [156].*

While the previously mentioned interactomes are all derived from Y2H experiments, there are also other means of generating large interaction networks. Two genome-wide tandem affinity purification (TAP) studies, where protein complexes were purified from yeast and identified by mass spectrometry (MS), were recently published [157, 158]. The benefit of pulldown experiments is the ability to detect both direct interactions in addition to indirect interaction (where two proteins are part of the same complex, but do not interact directly with each other). By identifying which complexes a particular protein is part of, one can get a better picture of which cellular processes the protein is involved in. Another possibility is to generate interaction networks by predicting PPIs in one organism based on experimentally observed interaction from a model organism. This method have been used to generate predicted human interaction networks, where *S. cerviciae*, *D. melanogaster* and *C. elegans* were used as model organisms [159]. Finally, it is also possible to generate networks based on

expression data [160]. By correlating how different genes are expressed during cell-cycle progression, or when stimulated with a specific substance, genes that show similar expression patters are indicated as having interactions between them. These transcription networks can give a good indication of genes which are involved in similar processes, which can then be further analyzed for direct interactions. The accuracy of expression correlation networks or other predicted networks can be increased by also including additional information, such as gene annotation and gene domain information [161].

The ever increasing amount of published protein-protein interactions have made it possible to text-mine the current literature, either manually or by using bioinformatic tools, to generate large interaction networks based on the results [162]. Individual interactions in these networks often have the benefit of being more thoroughly investigated, possibly resulting in a lower level of false positive interaction. These networks can however be somewhat biased towards proteins that have been more extensively studied, and does not necessary give a complete picture of interactions in a given organism.

### 1.2.4 Network topology

One common feature of cellular PPI networks is that a small portion of proteins have many interaction partners (so called hubs), while most proteins are only involved in a few interactions (Figure 1.6). What separates a PPI network from random distribution networks is the presence of these highly connected hubs, which tie together most of the proteins with only one or two interaction partners (Figure 1.7). The result is a very dense network where the distance between any two proteins (the number of edges one has to follow to get from one protein to another) is generally quite low. For the yeast network published by Schwikowski and colleagues, the average distance between any two proteins is only 3.05 [163]. Due to the generally small distance between any two proteins in these network, they are referred a being small-world networks. This phenomenon is not exclusive for cellular PPI networks, but was indeed initially observed in a study of the structure of the World Wide Web [164]. Later studies has determined that other networks, such as citation networks, food webs, metabolic networks and proteins-protein interaction networks share the same general network topology [155, 165, 166].

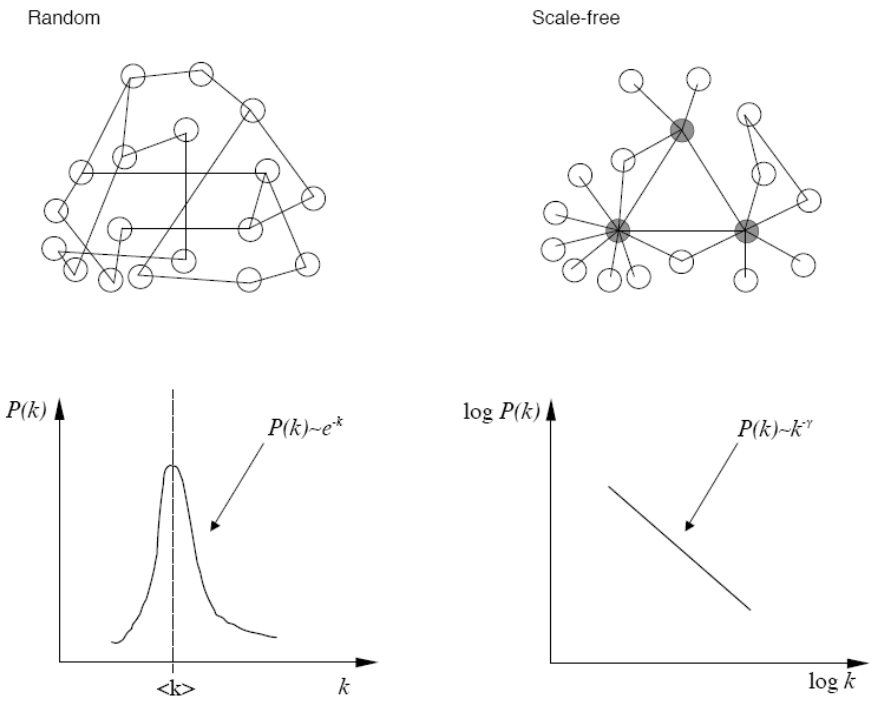


*Figure 1.7: Random vs scale-free degree distribution. For a randomly connected graph, the number of links for each protein p(k) follow a Poisson distribution where $p(k)\sim e^{-k}$. This graph has a clear peak (k), indicating the most common degree among its nodes. Scale-free networks however, do not have a clear peak in the distribution. While most nodes only have a few links, a few nodes have a large number of links (indicated in grey). The degree distribution of a scale-free network follow a power-law distribution where $p(k)\sim k^{-\gamma}$. Modified from [165, 167]*

These networks follow a scale-free topology, characterized by a degree distribution which follows a power-law relationship:

$$\mathbf{P(k) \sim k^{-\gamma}}$$

P(k) describes the number of proteins in the network which has a given degree (number of interactions), while γ indicates a coefficient which describes the decline of the graph (γ will vary between different networks) (Figure 1.7) [156]. The first cellular networks to be classified as having a scale-free topology was S. cerevisiae and Helicobacter pylori [156], and this topology has later been confirmed for most other cellular networks studied.

Cellular networks also tend to display modularity, with certain highly interconnected proteins forming sub-modules within the interaction network. These modules often correlate to specific complexes or groups of proteins with similar functions that have many interactions

between them. The subunits of the proteasome are one example of proteins that are highly interconnected which may stand out as a sub-module in the cellular network [168].

### 1.2.5 Extracting biological information from interactomes

With the increasing number of large-scale interactomes being published, there is a growing interest in analyzing these networks by different bioinformatical means. A large number of studies have described the topological properties of these networks in more detail. Although, it is yet unclear why cellular networks display a scale-free topology, one can speculate that it has some biological relevance for the cell. One possible explanation lies in the fact that scale-free networks have a relatively high tolerance against random failures (i.e. if one random protein should be taken out of the network) [169]. *In vivo* this could correlate to a given mutation rendering a protein unable to fulfill its original function, or being knocked down through siRNA interference. From studies in yeast it has been observed that the loss of a single gene will in most cases not be lethal to the organism [170, 171]. However, there are some proteins that are essential to the cell, where removal of one protein can have a profound effect on the cell. One of the hallmarks of scale-free networks is that, while tolerant to random failure, they are highly susceptible to targeted attacks. When the hubs of a scale-free network are removed one-by-one the overall network quickly collapses [169]. Similar finding have been reported for cellular network. In example, studies done in yeast have shown that there is a correlation between centrality (number of interactions) and lethality in the yeast PPI network, indicating that hubs have a higher probability of being essential [156, 172]. This finding has however been disputed in a separate study [173]. In addition, hubs have been suggested to have a higher probability of being involved in cancer development [174], and two separate studies has indicated that hubs tend to be more conserved than proteins with few interaction partners [175, 176].With hubs seemingly having such a central role in both interaction networks, and in the biology of the organisms, there is now an increased effort to discover what separates these proteins from other proteins. Several suggestion have already been proposed, such as hubs have higher intrinsic disorder (domain which are more loosely folded) [177-179], more sequence repeats [177] and higher portion charged residues on the protein surface domains [179]. Whether these are the only factors involved is more doubtful, and further studies will most likely reveal other, and possibly more important, differences between hubs and non-hubs.

As more interactomes are becoming available, the possibility of comparing these networks against each other has become more apparent. This has spawned the field of comparative interactomics which seeks to explore the similarities and differences of biological networks. This approach has been used to identify conserved interactions between different species [173, 180], and new bioinformatical tools now has the possibility to align different networks similar to what is generally done for amino acid or nucleotide sequences [181].

# 2 Aims of the study

Throughout the replication cycle of herpesviruses there are a multitude of protein-protein interactions taking place which ensures the production of new infectious viral particles. Gaining a better understanding of how the viral proteins interact with each other and with cellular proteins will help elucidate the details of the viral replication cycle, and possibly aid in the identification of new drug targets. The aims of this study will further be described in three paragraphs:

**I Intra-viral interactions**

Herpesviral proteins interact with each other at different stages during the infection (e.g. replication of viral DNA, capsid formation and viral egress). We aim to study intraviral protein-protein interactions in several species of herpesvirus on a genome-wide level using the yeast-two-hybrid system. To achieve this we will clone the complete ORFeomes of the five herpesviral species HSV-1, VZV, mCMV, EBV and KSHV, and analyze the proteins for the ability to interact with each other. Resulting interaction networks will be further processed using bioinformatical tools, and potentially interesting interaction will be studied in more detail.

**II Virus-host interactions**

Protein-protein interaction between viral and cellular protein will be studied for VZV and KSHV to better understand how herpesviruses are able to take control over a host cell. The two virus-host networks will be compared to identify common cellular targets for the two viruses, and to elucidate on general strategies which herpesviruses apply to gain control over a host cell.

**III Conserved interactions**

Using the large datasets of intraviral PPIs obtained, we wish to identify interactions that are conserved between closely and remotely related herpesvirus species (e.g within a subfamily and between subfamilies). Using bioinformatics tools we hope to identify specific factors which may help determine if interactions are conserved or not.

# 3 List of papers

**Paper I**

**Herpesviral Protein Networks and Their Interactions with the Human Proteome**

Peter Uetz, Yu-An Dong, Christine Zeretzke, Christine Atzler, Armin Baiker, Bonnie Berger, Seesandra V. Rajagopala, Maria Roupelieva, Dietlind Rose, **Even Fossum**, Jürgen Haas**.** *Science* **311**, 239 (2006)

**Paper II**

**Herpesviral proteins preferentially target highly connected human host proteins**

Yu-An Dong, Manfred Koegl, Ulrich Stelzl, Armin Baiker, Caroline C Friedel, Dietlind Rose, Petra Lutter, Albrecht von Brunn, Frank Schwarz, B Kasmapour, Erich E Wanker, Ulrich Koszinowsky, Ralpf Zimmer, Peter Uetz, **Even Fossum**, Jürgen Haas. Submitted

**Paper III**

**Comparative interactomics point to evolutionary conserved herpesviral protein-protein interaction networks.**

**Even Fossum**, Caroline C. Friedel, Silpa Suthram, Seesandra V. Rajagopala, Björn Titz, Armin Baiker Tina Schmidt, Theo Kraus, Sourav Bandyopadhyay, Dietlinde Rose, Mareike Uhlmann, Christine Zeretzke, Yu-An Dong, Hélène Boulet, Susanne M. Bailer, Ulrich Koszinowski, Trey Ideker, Peter Uetz, Ralf Zimmer and Jürgen Haas

# 4 Summary of papers

## *4.1 Paper I*

**Herpesviral Protein Networks and Their Interactions with the Human Proteome**

The first paper presents the genome-wide yeast-two-hybrid (Y2H) analysis of intraviral protein-protein interactions (PPI) in the two herpesviruses Kaposi's sarcoma associated herpesvirus (KSHV) and varicalla zoster virus (VZV). A total of 89 KSHV ORFs were cloned and analyzed for PPIs, revealing 123 interactions between 50 ORFs. For VZV, 69 ORFs were analyzed and 173 interactions observed between 59 ORFs. Approximately 50 % of the interactions detected in the Y2H screen for KSHV could be confirmed in an independent co-immunoprecipitation (CoIP) assay. While the viral network shared some characteristics with cellular networks, like having relatively many hubs (typical for a scale-free networks), they differed in other parameters. The most common number of interactions for the KSHV proteins was three, which is in contrast to cellular networks where one single interaction is the most common. Therefore, the herpesviral networks appear as highly connected modules, and display a power-law distribution that is divergent from known scale-free networks. Due to a lack of known virus-host interactions, the viral networks could not be integrated into a cellular network based on published interactions alone. In order to circumvent this problem, virus-host interactions were predicted based on homology to known interactions in *S. cerevisiae*, *C. elegans* and *D. melanogaster*. Altogether 20 virus-host interactions were predicted using this method, allowing the viral networks to be successfully connected into the human interaction network. From 19 of the predicted interactions, 13 could be confirmed by CoIP. As the predicted virus-host interactions were added to the viral network, the combined virus-host networks adapted to a scale-free topology which was more similar to other known cellular interaction networks. These findings suggest that while viral interaction networks differ from cellular networks when considered separately, they adapt a topology more similar to cellular networks once connected into a host network.

## 4.2 Paper II

**Herpesviral proteins preferentially target highly connected human host proteins**

This paper presents two Y2H screens identifying protein-protein interactions between viral and cellular proteins. For KSHV, the complete ORFeome (from Paper I) was analyzed against a cellular clone collection initially presented by Stelzl and colleagues [155]. From more than a million KSHV-host interactions analyzed in an all-against-all matrix approach, 252 interactions between 49 viral and 131 cellular proteins were observed. For VZV, a library Y2H analysis was performed, revealing 876 interactions between 61 viral and 755 cellular proteins. If more stringent criteria were used, a high confidence (HiFi) dataset of 154 interactions between 38 VZV and 145 cellular proteins were obtained. From the KSHV-host network, a subset of interactions was analyzed by co-immuno precipitation, and approximately 50% of the interactions could be confirmed. Although there was little overlap between the two virus-host networks, a set of 13 cellular proteins were targeted by both viruses, and two of these proteins were targeted by orthologous proteins in VZV and KSHV. While 12 of 13 common targets were never before reported to interact with any herpesvirus protein (RPA1 interacts with EBNA1 [182]), several of the proteins could be connected with different aspects of herpesvirus morphogenesis.

Bioinformatical analysis of the two virus-host networks indicated that the viral proteins preferentially interacted with highly connected cellular proteins (hubs). This tendency was observed for both VZV and KSHV, indicating that the targeting of hubs might be a common feature for herpesviruses. When comparing the number of virus-host interactions to the number of intraviral interactions (from Paper I) for each viral protein, it was observed that different viral proteins acted as hubs in the two networks. (i.e. proteins with many intraviral interactions generally had quite few virus-host interactions). Further, if the viral proteins were sub-divided into conserved (core) proteins and non-conserved (non-core) proteins, core proteins were more likely to interact with highly connected cellular proteins than non-core proteins. In addition, the core proteins were observed to have significantly more cellular interaction partners than the non-core proteins. Taken together, these finding present a well of new virus-host interactions for two species of herpesvirus, and an insight into the strategies herpesviruses use to gain control over a host cell.

## 4.3 Paper III

**Comparative interactomics point to evolutionary conserved herpesviral protein-protein interaction networks.**

This paper presents the genome-wide analysis of protein-protein interactions in HSV-1, mCMV and EBV. Altogether 941 intraviral PPIs were observed, including 113 interactions in HSV-1, 397 in mCMV and 190 in EBV. When these data were combined with the two networks presented in the first paper, a dataset of 1007 interaction in five different herpesvirus species were obtained. From 492 interactions between viral proteins conserved in more than one species, 137 were actually observed in two species or more. Although there were a significant number of conserved interactions between all the five species, only two interactions were observed in four species (and none in all five).

Herpesviruses encode 41 core proteins which are present throughout the three subfamilies. Of the 218 non-redundant interactions observed among these core proteins, 47 were detected in more than one species. There was however no correlation between the sequence similarity of a gene, and the number of species in which an interaction were confirmed. To evaluate if interaction among core proteins were conserved or species specific we predicted interactions in HSV-1, mCMV and EBV based on the interaction network of KSHV. A total of 92 predicted interactions were analyzed by CoIP, of which ~ 60% could be confirmed, indicating that many interactions among core protein are conserved. There was also a significant correlation between the number of interactions confirmed by CoIP, and the number of herpesviruses in which an Y2H interaction was observed. This suggests that the interactions observed in several species may be considered of higher confidence than interactions only observed in one species.

An interesting interaction between the mCMV proteins M51 and M53 was further evaluated by immuno-fluorescence using fluorescent fusion proteins. M51 was observed to co localize with both M53 and M50 in transiently transfected HeLa cells, possibly reflecting a functional connection between these proteins.

# 5 Discussion

## *5.1 General results and methodology*

The three papers presented here describe an extensive study of viral-viral and viral-host protein-protein interactions (PPI) using yeast-two-hybrid (Y2H) as the main experimental method. From the intraviral interaction networks of five different herpesvirus species it was observed that these networks differ topologically from cellular networks. However, when two of the viral networks were connected to a cellular network, through predicted interactions, the combined networks adopted a topology similar to previously published cellular networks. Although there was little overlap between the five viral networks, an unrooted phylogenetic three (based on the neighbor joining algorithm) could still be obtained using PPI as the only data source. Further, the generation of a core network, where interactions among core herpesviral proteins from all five viruses were overlaid, allowed the extraction of interesting interactions which were not apparent in each single network. Two separate genome-wide studies of virus-host PPI for KSHV and VZV indicate that there are common cellular targets between the two viruses, and suggests that herpesviral proteins preferentially interact with highly connected cellular proteins (hubs).

All three studies presented here rely heavily on Y2H as a method for detecting protein-protein interactions. As indicated in the introduction, this method is error-prone with relatively high numbers of false positive and false negative interactions. There are however means for addressing these problems in order to obtain more reliable data. Some proteins, mostly bait-proteins, are known to be able to promote the transcription of the reporter gene without the presence of an interacting prey-protein. These proteins are referred to as self-activators, and can be a major source of false positive interactions. Using a chemical inhibitor (3-amino-1, 2, 4-triazole (3-AT) (Invitrogen)), which inhibits histidine biosynthesis [183], it is possible lower the self-activation of these proteins [184]. In the studies presented here, all viral bait proteins were tested for self-activation, and self-activating baits were further analyzed on rising concentrations of 3-AT. The proteins which displayed self-activation on high concentrations of 3-AT, were taken out of the results. Another commonly used method for increasing the specificity of the Y2H results is to test interactions more than once. This can either be done by retesting observed interactions, or testing each interaction in duplicates/quadruplicates. For the five intraviral networks, all interactions were tested in

quadruplicates, with the exception of mCMV which was tested in duplicates. The KSHV-host interaction analysis was based on a matrix Y2H approach, and all interactions observed in the initial analysis were retested, and only interactions confirmed in the retesting were included in the results. For the VZV-host analysis, a library Y2H approach was undertaken, and all observed interactions were included in the VZV-all dataset, including interactions only observed once. However, interactions observed more than once were considered to be of higher confidence, and make up the VZV-HiFi dataset (Paper II). Since all the interactions presented in these three studies, with the exception of the VZV-all dataset, have been observed at least twice, we believe that the false positives rate have been brought down to a reasonable level. From the KSHV intraviral, and KSHV virus-host studies, approximately 50% of the observed interactions could be reproduced using CoIPs as a second method, which is in accordance with similar publications [155, 185]. Since our CoIP system is based on over-expressing tagged proteins using Vaccinia virus, it is possible that this system also is burdened with false positive and false negative interactions. However, in Paper III (Supplemental Figure 10) we observed a good correlation between conserved core interactions detected by Y2H, and core interactions confirmed by CoIP. From this finding we conclude that the two methods support each other, and that interactions observed by both Y2H and CoIP most likely are true positives.

Determining the false negative rate is however more difficult. Paper III indicate that there is in general little overlap between the five virus networks, which is also seen for other large interactome studies [143, 144]. In contrary, analysis of the predicted interactions indicates that there is a considerable amount of conserved interactions, which is not evident from the Y2H studies alone. Also, very few of the PPIs known from literature were detected in our Y2H studies, once again indicating that the studies have a high false negative rate. Based on the CoIP data it is possible to stipulate a false negative rate ranging from 59 - 78 %, when looking at the difference between predicted interactions confirmed by CoIP and those confirmed by Y2H. If we however use the previous published interactions as a reference, the false negative rate is 63 – 90 % for the intraviral studies (VZV and EBV was not included since no published interactions were detected). While these numbers have a high degree of uncertainty, they indicate that there are still a large number of intraviral PPIs which remain to be detected. From our virus-host study, only one published interaction was detected, indicating that the false negative rate in this screen was even higher than in the intraviral studies.

Another problem connected to interactions obtained from Y2H studies is that they are not separate in time and space. Some interactions might be limited to specific intracellular compartments, or to given time points. Especially for viral infections it would be of interest to know if a given interaction occurs right after the virus has infected a host cell, or later during the production of new infectious particles. By combining interactome data with microarray data, describing when certain genes are expressed, it is possible to obtain a better understanding of when specific interactions may occur in the cell. Supplemental tables S16 – S19 in Paper II summarizes the combination of KSHV-host interactions with expression profiles of the viral ORFs derived from microarray studies by Jenner and colleagues [186]. By separating the viral ORFs into three different expression classes, it was possible to examine if some cellular proteins were mainly targeted by viral ORFs within one specific expression class. Although this was the case for some genes (i.e. KRBA1, EMD and MRLP17), one can not entirely exclude that there is overlap in the expression profile of the three classes. Early genes and immediate early genes may be expressed throughout the viral cycle. In addition, structural proteins expressed late in infection will be part of newly formed infections particles, and their interactions may be associated with the initial takeover of a new host cell. More detailed studies are therefore necessary to draw more robust conclusions regarding when different interactions might take place during an infection.

## *5.2 Biological relevance of experimental results*

Protein-protein interactions define literally all important aspects of the herpesviral biology, ranging form the initial attachment to the host cell, to assembly and release of newly formed virus particles. Here we present a massive number of newly discovered intraviral and virus-host interactions. While it is impossible to go into detail on all of these novel interactions, the observed PPIs have revealed general aspects of intraviral interactions and of how virus proteins interact with cellular proteins.

Paper I suggests that intraviral PPI networks are topologically different from cellular PPI networks. While cellular networks are scale-free, and display a high degree of local clustering, this is not the case for viral networks. As previously mentioned, having a scale-free topology might be beneficial for the cell due to the tolerance towards random failure [169]. Whether there is any biological relevance in intraviral networks having a different

topology is however less certain. Intraviral PPI networks represent a combination of interactions that take place within the virusparticle and at different time points within the infected cell. Also, when considering that a virus is literally a lifeless particle without its host, the resulting intraviral network does not really reflect any actual model of an in-vivo situation. However, if these networks are combined with virus-host interactions, the resulting combined network may give a better representation of the network topology of an infected cell. Interestingly, in Paper I we observed that the intraviral network adapts to the topology of the cellular network when connected through predicted virus-host interactions. Further studies into the topology of combined pathogen-host networks may allow the differentiation of a healthy cell and an infected cell, based only on the properties of the interaction networks.

In Paper II we reported that viral proteins tend to interact with cellular hubs, and that this holds true for both the KSHV-host and the VZV-host analysis. This finding was also recently reported in another virus-host Y2H study, between EBV ORFs and cellular proteins [185]. Since the same conclusion has been drawn for three separate herpesviruses, in two different studies, it is plausible that the targeting of hubs is a common phenomenon in herpesvirus-host interactomes. From a topological point-of-view, scale-free networks (like cellular networks) are tolerant to random failure, but highly susceptible to targeted attacks directed at hubs [169]. Targeting hubs may thus be the virus' strategy for maximizing their impact on the host network. Also from a more biological perspective it makes sense for the virus to target highly connected proteins. Since herpesviruses affect a large variety of cellular functions, like transcription, proteasome activity and cytoskeletal rearrangement [104, 187], it is more efficient for the virus to target central components for each of these functions.

Paper III indicates that a substantial portion of interactions among core herpesviral proteins are conserved, despite generally low sequence similarity. From literature there are several examples of interactions between core proteins that are conserved among several herpesvirus species from different subfamilies; e.g. HSV-1 UL31 and UL34 [93, 94, 188], HSV-1 UL54 self-interaction [189, 190], HSV-1 UL15 and UL28 [89, 191]. Indeed much of what is currently known about herpevirus biology is derived from studies done in Herpes Simplex, which are assumed to hold true for other species as well. Other studies has indicated that it is possible to transfer PPIs from one species onto another [173, 180], which seems to be in accordance with what we see for herpesviral interactions. There have also been attempts to study the level of sequence similarity needed to confidently transfer interactions from one

species onto another. Yu and colleagues observed that interactions could be confidently transferred from one species to another if the joint sequence similarity of the interacting orthologs were > 80% [192]. However, Paper III indicate that the transfer of interactions between herpesvirus species must be somewhat different since none of the core proteins studied share such a high degree of sequence similarity. Indeed, we observed no correlation between sequence similarity and the number of species in which an interaction was conserved. From our finding it is clear that a high degree of sequence similarity is a poor criteria when predicting interactions from one species onto another, at least in the case of viruses.

When comparing the number of intraviral and virus-host interactions from both core and non-core proteins, it was observed that core proteins tended to have more host interactions (Paper II). This was somewhat surprising since most of the herpesviral genes reported to be involved in virus-host interactions, e.g. immune-evasion or anti apoptotic functions, are indeed species specific proteins [193]. The result may on the other hand give an indication of why conserved proteins are conserved. Since the mutation rate is higher in herpesviruses than in mammals [194], a large number of host interactions might result in an evolutionary pressure against mutation in core proteins.

## *5.3 Specific interactions of interest*

In the three studies presented here 1007 intraviral and 1128 virus-host interactions have been reported. From the intraviral interactions 47 was observed in more than one species, reflecting possible conservation across different species of herpesvirus. With such a large number of interactions observed it is impossible to examine all of them in detail, but we can however speculate on which function they might have during the viral replication cycle. In this section some of the more interesting interactions will be discussed in detail and in relation to what is know from literature.

### 5.3.1 Function of M51

mCMV M51, and its orthologs HSV-1 UL33, VZV ORF 25, EBV BFRF4 and KSHV ORF 67.5, were observed to have a number of interesting interactions in the intraviral Y2H screens. From the overlay network presented in Paper III the interaction between M51 and M53 was observed in four different species, with HSV-1 being the only species where it was not

observed. Interestingly, from the retesting of UL33 interaction on rising concentrations of the auto-activation inhibitor 3-AT (Paper III, Supplementary Figure 13), the interaction between UL33-UL31 stands out as one of the positive interactions. These results were however not included in the HSV interactome, to prevent an overrepresentation of interactions tested more than once. M53 has been extensively documented to be involved in nuclear egress through its binding to M50 [94, 195-197]. M51 was observed to interact with M50 in one species (VZV), although also this interaction stands out as positive in the UL33 retesting. In addition to Y2H, immunofluorecense studies indicated that M51 co-localize with both M53 and M50 when using fluorescent fusion proteins. From these result one might speculate that M51 plays a role in nuclear egress, through interactions with M53 and/or M50.

Previous studies have indicated that HSV-1 UL33, the ortholog of mCMV M51, is involved in packaging of DNA. HSV-1 strains with temperature sensitive mutations within the UL33 gene was shown to be incapable of proper cleavage and packaging of concatomeric DNA into preformed capsids [88]. Other studies have suggested that UL33 is probably more involved in cleavage of DNA due to its ability to interact with subunits in the terminase complex (UL28 and UL15) [90]. A recent report have suggested that UL33 is necessary to stabilize the complex of UL28 and UL15 [91]. In the Y2H studies presented here UL33 was observed to interact with UL15 in three different species (not including HSV-1), while the interaction between UL33 and UL28 was observed in 2 species (HSV-1 included). These results seem to correlate well with what has been previously published for HSV, also indicating that these interactions might be conserved throughout the herpesvirus subfamilies. It also exemplifies that by generating an overlap network from several genome-wide Y2H screens in related species, it is possible to address the high number of false negative interactions within each individual analysis.

When combining the previously mentioned interaction between M51 and M53/M50 with published interactions between UL33 and UL28/UL15, one might speculate whether M51 is involved in both packaging and egress. From studies done in HSV it was shown that UL33 is associated with capsids during packaging of DNA and that this association is independent of UL28 and UL15 [198]. Later studies by the same group have indicated that UL33 is located on the external surface of capsids [199]. To my knowledge, no proteins has been identified to attach UL33 to the capsid. However, in the mCMV Y2H analysis M51 was observed to interact with the smallest capsid protein m48.2. Whether this holds true for HSV is less

certain, but it would fit the description of UL33 attaching to the surface of the capsid. From immunofluorecense studies it has been shown that HSV-1 UL33 is located within nuclear replication compartments of infected cells late in infection [200]. However, Yamauchi and colleagues showed that when HSV-2 UL33 was transiently transfected into cells it displayed a cytoplasmic staining pattern [201]. This is similar to what was observed for M51-YFP fusion proteins transfected into HeLa cells (Paper III). Yamauchi and colleagues could also show that co-transfection with UL14 resulted in a re-localization of UL33 into the nucleus. From our Y2H studies the orthologs of UL33 was observed to interact with UL14 in two species (mCMV and KSHV). Additional experiments, not included in Paper III, could show that M51-EGFP fusion proteins was translocated to the nucleus of HeLa cells when co-transfected with M95-DsRed2 (Figure 5.1). From the immunofluoresence studies, the Y2H analysis and the previous publications it seems likely that UL14 translocation of UL33 represent a conserved function in herpesviruses.

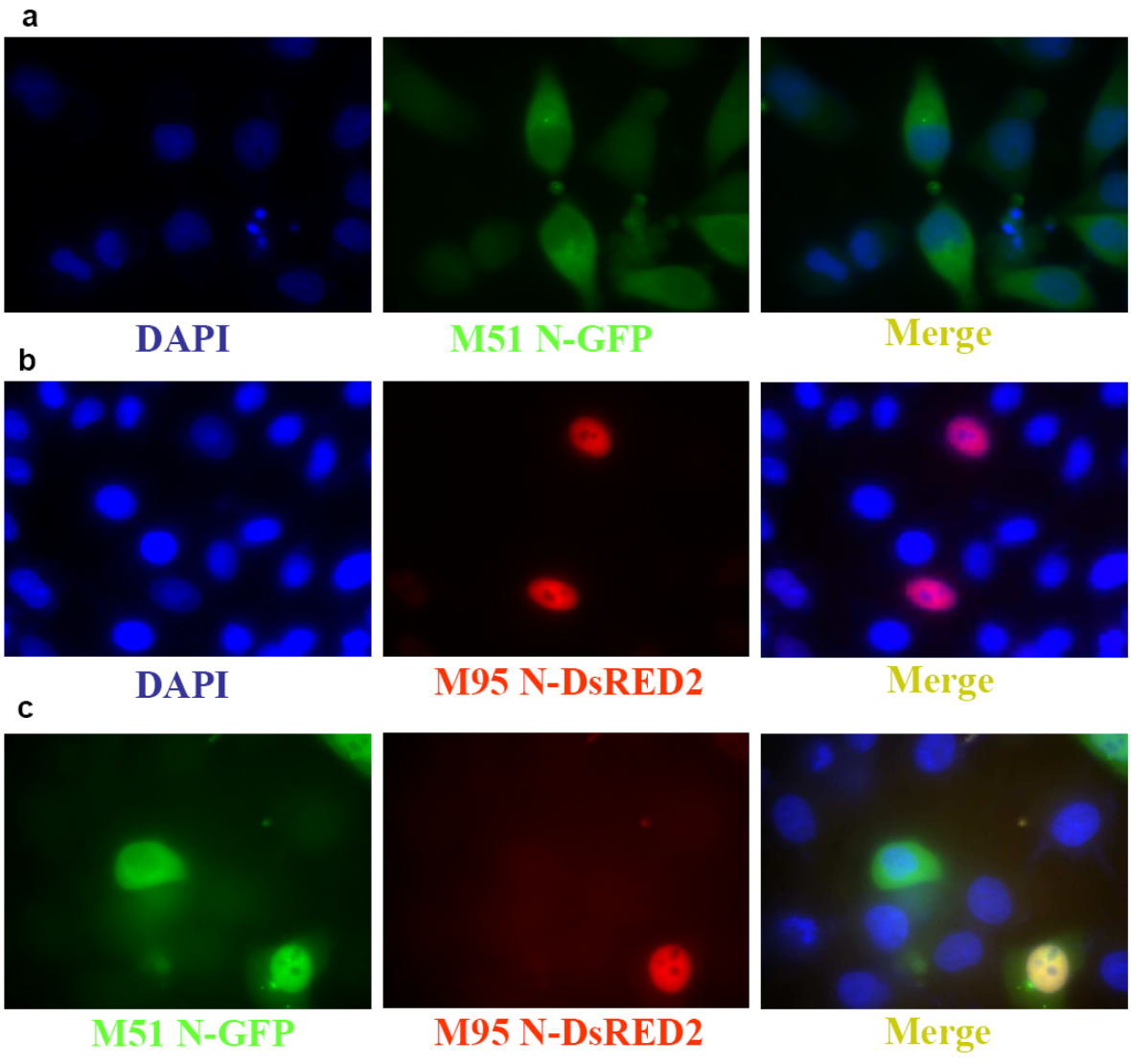

*Figure 5.1: M95 translocates M51 into the nucleus of transiently transfected HeLa cells. A) While M51-GFP (M51 fused N-terminally to EGFP) transfected into HeLa cells gives a cytoplasmic staining patter, b) M95-DsRed2 gives a clear nuclear staining pattern. C) Co-transfecting M51-GFP and M95-DsRed2 however leads to a relocalization of M51-GFP into the nucleus of the cell.*

### 5.3.2 Other interactions of interest

DCTN1 (dynactin 1, p150 subunit) is the large subunit of the dynactin complex involved in retrograde transport along microtubule. Herpes Simplex virus capsids have been reported to use dynein and dynactin for efficient transport from the cell periphery to the nucleus [78]. Blocking dynactin activity inhibits this transport [202], and it is believed that viral protein(s) interacting with dynactin are located within the inner tegument layer [203]. KSHV has also been reported to use dynein motors for transport to along microtubule, although the specific viral or cellular proteins involved in the process remains to be identified [79]. From Paper II it was observed that both VZV and KSHV interacted with DCTN1. Indeed two separate viral proteins in each virus interacted with DCTN1, although these proteins were not othologous between the two viruses. When considering the published results for HSV-1 together with the interactions presented here, one might speculate that the use of dynein and dynactin represents a common strategy of herpesviruses for transporting capsids to the nucleus.

IKBKAP (inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase complex-associated protein) is a part of the NF-κB pathway, as an upstream regulator of NF-κB-inducing kinase (NIK) and IκB kinases (IKK) [204]. NF-κB activation has been reported to play an important role in the pathogenesis of several herpesviruses, with several reports claiming it is essential for viral replication [205, 206]. However, viral proteins have also been reported to inhibit NF-κB activation as an immune evasive strategy [207]. Interestingly, KSHV ORF K10.5 (which we observed to interact with IKBKAP) has previously been reported to inhibit NF-κB activation through binding to IKKβ [208]. This binding was observed *in vitro*, but failed to be reproduced in KSHV infected-BCBL cells. One can speculate whether other cellular proteins are involved in stabilizing the interaction. While there are no viral proteins known to inhibit NF-κB activation in VZV, HSV-1 ICP27 has been

suggested as a candidate to modulate NF-κB activation in as a means of inhibiting cytokine expression [209].

The KSHV protein ORF19 gave 35 interactions in the KSHV-host analysis, and its ortholog VZV ORF34 gave 7 interactions in the VZV-host analysis (VZV-all). When going into more detail on the different interaction partners of ORF19 it was observed that several of them have been reported to be involved in functions related to chromatin remodeling and transcriptional regulation. Interacting proteins such as MYST2 and HTATIP are both histone acetyltransferases [210, 211], whereas SETDB1 is a histone methyl transferase [212]. ORF19 also interacted with CBX5 (Heterochromatin binding protein1 (HP1)) which is involved in transcriptional silencing [113], and ING5 which have been reported to involved in regulation of chromatin acetylation and gene expression [213]. In addition ORF19 interacted with MCM3AP which is an acetyl tranferase involved in DNA replication [214] and SP110 (nuclear body protein) which may act as activator of gene transcription [215]. From these interactions one might speculate that ORF19 is involved in transcriptional regulation of viral and/or cellular genes. From the VZV-host analysis, VZV ORF34 was observed to interact with two different variants of histone H2A, also suggesting a possible connection to DNA and chromatin. However, from literature UL25 was initially reported to be required for encapsidation of DNA into capsids in HSV-1 [216]. These results have however later been disputed in studies which indicate that low levels of HSV-1 DNA could still be packaged in UL25 mutant viruses [217]. A separate study from Ogasawara and colleagues has indicated that UL25 is able to bind DNA [218], and several studies has indicated that UL25 is associated with mature capsid particles [218-220]. The fact that UL25 is able to bind DNA could be interesting when combined with the cellular interactors involved in gene regulation and chromatin remodeling, and may open for further studies into the function of this protein.

## *5.4 Future validation of interactions*

As previously mentioned the large number of interactions presented in these studies makes it difficult to validate or retest all of them. The general approaches of large scale interactome studies have been to retest a subset of interactions, and then to extrapolate from these results an estimate of the false positive rate. In the studies presented here co-immunoprecipitation has been used as a second method to evaluate the outcome of the Y2H analysis. This technique is however far too laborious for retesting all interactions observed. Further development of

reliable methods for retesting interactions in a more “intermediate-throughput” fashion is therefore needed. Such studies are indeed in progress with several promising novel methods for detecting protein-protein in mammalian cells, including LUMIER (luminescence-based mammalian interactome mapping) [221], FRET (Förster resonance energy transfer) [222] in addition to protein complementation assays like the β-lactamase assay [223]. While these techniques are promising they also have specific limitations, like the need for expensive equipment and difficulties in establishing the procedures. So the need for easy-to-use and moderately priced methods is still present.

High content screening is another field which has been in rapid development over the last few years. By combining microscopy (live-cell, immunofluoresence and/or bright field) and high-throughput automation it is possible to obtain large quantities of data, either from monitoring the growth and development of living cells exposed to different conditions, or through imaging of fixed cells. These types of studies produce a large quantity of images which can be processed by specific software to identify differences in cell morphology, viability or sub-cellular localization of labeled proteins. This technology represents one possible method for doing different functional studies of herpesviral proteins on a genome-wide scale. For example studies of cellular localization, both of viral proteins and of cellular proteins, has already been published [224, 225], suggesting that similar studies may be performed on herpesviruses. There has also been a recent publication where high content screening has been used to study siRNA knock down of cellular proteins on a larger scale. Neumann and colleagues used siRNAs against 49 different cellular targets to study which proteins are were involved in chromosome segregation [226]. One can imagine that a similar approach could be used to study the effect of knocking down different cellular genes on the replication of a herpesvirus, thus giving an indication of which cellular genes are essential for the virus to efficiently replicate. These results could then be correlated with e.g. interactome data in an attempt to identify essential virus-host interactions.

In addition to helping our understanding of the basic biology of herpesviruses, these large scale interactome studies can be beneficial from a clinical viewpoint. As indicated in the introduction, herpesviruses are connected to a variety of diseases in man. Any knowledge that could be used to improve drugs or disease treatment would therefore be helpful. One such possibility is extracting potential new targets for antiviral drugs from the protein-protein interactions. Especially the intraviral interactions that seem to be conserved throughout the

herpesviridae may be interesting targets for small-molecule inhibitor screens, where one chemical might have an inhibitory effect on several species of herpesviruses. This also holds true for the cellular targets. Any interaction with cellular genes that are essential for virus replication may prove valuable in the search for new forms of medication. There is however needs to further verify and study these interactions before moving on to the search for possible inhibitors.

## 5.5 Concluding remarks

Through the data presented here it is apparent that genome-wide interactome studies of both intraviral and virus-host interactions are not only feasible, but also provides a large amount of novel data. While single interactions of biological interest can be manually extracted from the data, further bio-informatical processing allows us to make observations and hypothesizes of a more general nature. However, since the fields of interactomics and system biology in general are quite new, there is a limited amount of publications available. This holds especially true for herpesviral interactomics where there are so far only two published papers available. One presented here (Paper I), in addition to the publications by Calderwood et al. [185] (which incidentally was published during the final preparations of Paper II and III). The lack of literature has made correlating our findings to previous publications difficult, and while on one hand we can claim that much of the data presented here is truly novel, further independent studies will need to confirm our findings. It is therefore comforting that one of the observations done for the virus-host networks, that viral proteins target hubs, was also observed in the EBV-host network from Calderwood et al.[185]. As more interactomes becomes available, for both viruses and other pathogens, it will become possible to further assess the validity of our other findings.

# 6 References


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